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# SUCCESSIVE STATISTICAL APPROACHES FOR OPTIMIZATION OF ANTIBIOTIC PRODUCTION BY *NOCARDIOPSIS PRASINA* ACT24

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## ABSTRACT

Production of secondary metabolites by microorganisms differs qualitatively and quantitatively depending on the strains and species of microorganisms used as well as on their nutritional and cultural conditions. The application of a statistical experimental design approach for the optimization of nutritional factors is one of the recent techniques that have been successfully applied to the production of various bio products. In this study we attempted to select prominent nutrient for antibiotic production by using Plackett–Burman design then further optimized the concentration based on central composite design to improve the active ingredient. In order to improve the yield of antibiotic by the new strain, *Nocardiopsis prasina* ACT 24 response surface methodology was employed to optimize the composition of fermentation medium. The Plackett-Burman design indicated that pH, agitation, incubation time and starch had significant effects on antibiotic production. The concentrations of these five components were investigated using Box-Behnken design and a polynomial model related to medium components concentration effect on antibiotic yield had been established. The factors optimized in the present study were useful for the increased production of antibacterial metabolite from *Nocardiopsis prasina ACT24.* The zone of inhibition was improved from 27mm to 30mm. The present investigation will be useful for large scale fermentation in a fermenter for the efficient production of antibiotic.

**KEYWORDS:** ***Nocardiopsis, Antibiotic, Fermentation, Statistical design and Optimization.***

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# INTRODUCTION

The filamentous bacteria of the order Actinomycetales (actinomycetes) produced more than 9000 biologically active molecules out of which more than 60 pharmaceutical agents have widely used in the field of medicine[1]. Especially *Streptomycetes,* have been shown to be a prime source of antibiotics. However, the likelihood of finding novel compounds has dwindled due to extensive studies on the ubiquitous species. The mycelium of actinomycetes can elongate and branch unlimitedly. Bioactive metabolites from actinobacteria are commonly produced by submerged or solid state fermentation with one or two commonly used media. [2]. Media components and their optimum levels are critical to the secondary metabolites produced by microorganisms. In the field of antibiotics, much effort was directed toward optimizing production rates and directing the product spectrum. The optimization experiments are usually performed using non statistical one-factor-at-a-time and statistical experimental design approaches[3]. The application of a statistical experimental design approach for the optimization of nutritional factors is one of the recent techniques that have been successfully applied to the production of various bio products. Response surface methodology (RSM) is a compilation of mathematical and statistical techniques useful for emergent, improving, and optimizing processes. It is a well-known method applied in the optimization of composition in the medium and other critical variables responsible for the fabrication of biological molecules[4]. RSM can be used to evaluate the relative significance of several affecting factors even in presence of complex interaction. RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously[5]. In this study we attempted to select prominent nutrient for antibiotic production by using Plackett–Burman design then further optimized the concentration based on central composite design to improve the active ingredient content, reduce costs and enhance the antibiotic real production.

# MATERIALS AND METHODS

### *Organisms*

*Nocardiopsis prasina* ACT24 was isolated from marine soil sample from east coast of Pichavaram, Cuddalore (dt) of Tamilnadu and serially diluted and plated on Actinomycetes isolation agar. *Nocardiopsis prasina* was maintained on starch casein medium .They were stored at 4°C until required. Vancomycin resistant enterococcus sps were isolated from urine sample of ICU patient and directly inoculated in to VRE isolation agar and incubated at 37ºC for 24 hrs. They were stored at 4°C until required.

### *Batch Fermentation*

Stored strain of *Nocardiopsis prasina* ACT24 was inoculated in modified Synthetic Krasilvikov's medium cultivated for 4 days at 28°C in incubator and then inoculated into sterile medium in 250 ml flasks. The flasks were incubated in the dark at 28°C on a rotary shaker at 180 rpm $min^{-1}$ for 10d. The cultures were centrifuged (14,000 rpm, 15 min, 4°C) to separate the Actinomycetes cells and the supernatants. The supernatants were filtered through 0.22µm bacterial filter and stored at 4°C until required[6].

### *Antibiotic Activity Assay*

Antibiotic activity was measured by assaying the growth inhibition rate of test pathogen *Vancomycin resistant Enterococcus*. Briefly, 1mL sterile fermentation filtrate mixed with 9 ml Muller Hinton Agar were replaced in a dish or without sterile fermentation filtrate as control. After the medium solidified and inoculated, discs loaded with 24hrs culture and placed on the agar plate. The dishes were incubated in the dark at 28°C in incubator for 24 hrs and measured colony diameter of *Vancomycin resistant enterococcus.* In previous study the authors confirmed that the size of the zones of inhibition can be considered as measure of antibiotic titre[7-8].

### *Experimental design and data analysis*

In order to obtain the most influential factors for antibiotic production, various physical parameters are pH, temperature, glucose, starch, potassium nitrate, tryptone, inoculums size, agitation, NaCl and incubation time were evaluated by 'one-at-a-time' approach[9].The best carbon source was glucose and the concentration was 2% .The preliminary experiments revealed that the nitrogen source including yeast extract, meat extract were suitable for the production of the strain in some degree; the potassium nitrate were influencing the *Nocardiopsis prasina* antibiotic production .

### *Plackett-Burman design*

A Plackett-Burman design was used to screening the most significant fermentation parameters affecting the new strain antibiotic production[10].For the selection of various variables, "Design Expert 9.0.3.1" (Stat-Ease Inc, Minneapolis, USA) was used to generate and analyze the experimental design of Plackett-Burman. Plackett-Burman design (PBD) was employed for screening the most significant medium components for growth and antimicrobial compound production by *Nocardiopsis prasina* ACT 24. In the experimental design, the factors pH, temperature, glucose, starch, potassium nitrate, tryptone, inoculums size, agitation, NaCl and incubation time (independent variables) were screened by representing them at two levels, low (−) and high (+) in 12 trials. Table 1 shows media components, symbol code, and actual low and high level of the variables. Table 2 shows the detail of the design, each row represents a trial, and each column represents an independent variable. Finally Box-Behnken design and response surface methodology were further adopted to derive a statistical model for optimizing the medium components for antibiotic production.

### *Box-Behnken design*

Further medium optimization by RSM was concerned with the four (pH, starch, incubation time and agitation) important components ($P < 0.05$) and the negative factors were removed. Box-Behnken in Minitab 15.0 was used to optimize the concentration of the five factors[11]. The effect of each variable was calculated using the

following equation: E = (ΣM+ M−)/N. Where E is the effect of tested variable, M + and M – are responses of trials at which the parameter was at its higher and lower levels respectively and N is the number of experiments carried out. The standard error (SE) of the variables were the square root of variance and the significance level (p-value) of each variables calculated by using Student's t-test. t = Exi/SE, where Exi is the effect of tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

***Central composite design***

In order to quantify the influence of the selected independent variables, specifically starch concentration, agitation, incubation time and pH, on the responses (concentrations of antibiotic), a 22 central composite design with four coded levels leading to 14 experiments was used, which contained a factorial or fractional factorial matrix with center points and star points to allow estimation of the curvature [12].According to this design, the total number of treatment combinations is 2k + 2k + n0 where 'k'is the number of independent variables and n0 the number of repetitions of the experiments at the center point. For statistical calculation, the variables Xi have been coded as xi according to the following transformation: xi = Xi – X0 /δx. Where: Xi is dimensionless coded value of the variable. Xi, X0 the value of the Xi at the center point, and δx is the step change. A 2k-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments were employed for optimizing the medium components[13].

***Validation of the model***

The combination of different five experimental combinations of optimized variables, which yielded the maximum response, was experimentally validated by culturing *Nocardiopsis prasina* ACT 24 in optimized production medium. The statistical model was validated with respect to all significant variables within the design space.

## RESULTS AND DICUSSION

The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of Plackett-Burman experimental design. The main effect was calculated as the difference between the average of measurements made at the high level setting (+) and the average of measurements observed at the low level setting (-) of each factor. Figure 1 shows the Pareto chart for the effect of selected nineteen factors on antibiotic production.

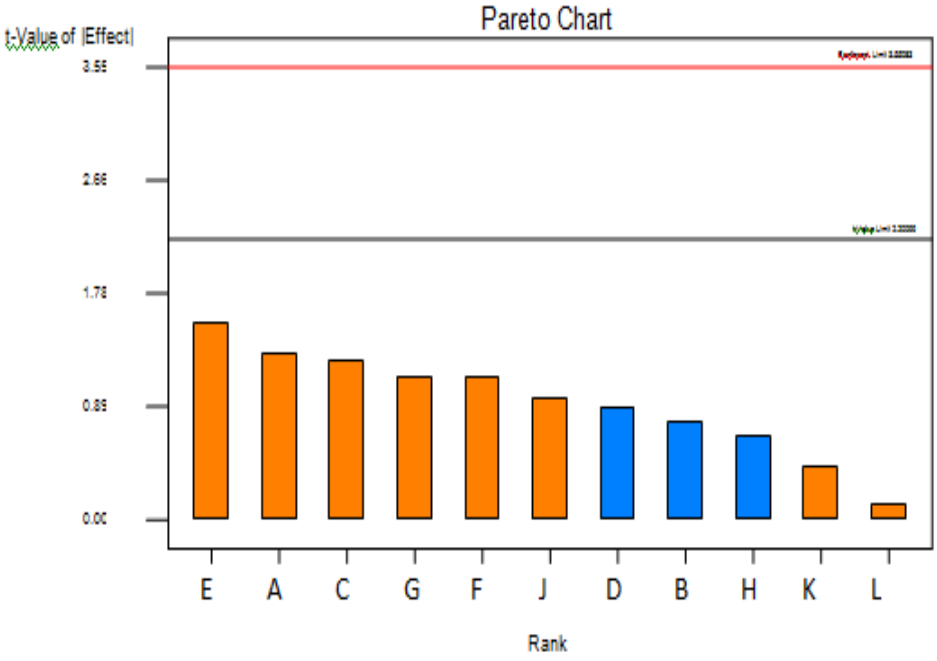



**Figure 1**
***Pareto chart showing the effect of the selected nineteen factors on antibiotic production***

A-pH, B-Temperature, C-Incubation time, D-Inoculam size, E-Agitation, F-Glucose, G-Starch, H-Pottasium nitrate, J-Tryptone, K-NaCl and L- Dummy. To improve the antibiotic production, *Nocardiopsis prasina* was investigated under different culture conditions such as pH, temperature, glucose, tryptone, Nacl, inoculam size, agitation, starch, potassium nitrate and incubation time. In the present study, the respective low and high levels with the coded levels in parentheses for factors were defined. Small manipulations in the culture medium composition can exert significant effect on secondary metabolites biosynthesis in microorganisms. The Plackett-Burman design has proven to be a valuable tool in screening and optimizing of media components and culture conditions in various bioprocesses including antibiotic production.

***Plackett-Burman design***

Plackett-Burman design has been applied by several researchers to select influencing factors among the

constituents of complex medium. Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of factors in a few experiments; it also saves time and maintains convincing information on each component [5- 6]. Plackett-Burman design for 10 selected variables and the corresponding response for antibiotic production. Variations ranging from 18 to 27 mm in the production of antibiotic in the 12 trials were observed by Plackett-Burman design. Pareto chart illustrates the order of significance of the variable affecting antibiotic production (Figure-1). The Plackett-Burman experimental design was adopted with 12 trials to determine the medium components which significantly influence the antibiotic production by *Nonomuraea* sp. JAJ18[14].Among the variables screened, the most reliable factors with high significance level indicated by Pareto chart were in the order agitation, pH, incubation time, Starch, glucose and tryptone starch showed a remarkable support for the growth of *Nocardiopsis prasina*. Incubation time, pH, starch and agitation were also identified as most potent significant variables in antibiotic production and selected for further optimization while inoculam size, temperature, tryptone and potassium nitrate concentration which exhibited less significant level were omitted in further experiments. Similarly, the author reported that factors varied depending on the type of used media[15]. The strain hydrolysed starch and liquefied gelatine. Several researchers working on antibiotics discovery programs have applied PBD and RSM as statistical tools to recognize, manipulate and optimize influencing medium constituents and recorded the increased antibiotic production[16-17]. Statistical analysis of the Plackett-Burman design demonstrated that the model F value of 6.05 is significant. The value of $p < 0.05$ indicate model terms are significant .The results of PBD revealed that the crucial media components related to the antibiotic production by *Nocardiopsis prasina* were starch. Similarly, the author reported that the antibiotic production by JAJ06 were starch, KBr, and $CaCO_3$[18].

**Table 1**
***Plackett-Burman experiments design for evaluating factors influencing and antibiotic production by Nocardiopsis prasina.***

| Code | Variables | Units | Levels | |
|---|---|---|---|---|
| | | | Low | High |
| A | Ph | - | 5 | 9 |
| B | Temperature | degree | 25 | 40 |
| C | Incubation time | days | 5 | 14 |
| D | Inoculum size | % | 0.5 | 2 |
| E | Agitation | rpm | 50 | 150 |
| F | Glucose | % | 0.5 | 2 |
| G | Starch | % | 0.5 | 2 |
| H | Potassium nitrate | % | 0.001 | 0.005 |
| J | Tryptone | % | 0.5 | 2 |
| K. | Nacl | % | 0.5 | 2 |
| L | dummy1 | - | -1 | 1 |

Planckket and Burman experiments and their levels were further optimized for enhanced antibiotic production by employing a Box – Behnken design and central composite design. Finally, the physical factors pH, agitation, starch and incubation time for each run, the experimental responses along with the predicted response obtained from the regression equation for the 29 combinations are shown in Table-2.

**Table 2**
**Box- Behnken design matrix of predicted and actual values of antibiotic production by *Nocardiopsis prasina***

| Run | Factor-1 A-pH | Factor-2 B- Agitation (Rpm) | Factor-3 C- Incubation time(Days) | Factor-4 D-Starch(%) | Antibiotic activity (mm) | |
|---|---|---|---|---|---|---|
| | | | | | Predicted value | Observed value |
| 1 | 9 | 100 | 14 | 1.25 | 27 | 25 |
| 2 | 7 | 100 | 14 | 2 | 28 | 27 |
| 3 | 7 | 50 | 12 | 0.5 | 26 | 24 |
| 4 | 7 | 50 | 14 | 1.25 | 26 | 25 |
| 5 | 7 | 100 | 12 | 1.25 | 27 | 26 |
| 6 | 5 | 100 | 14 | 1.25 | 23 | 21 |
| 7 | 9 | 150 | 12 | 1.25 | 28 | 27 |
| 8 | 9 | 100 | 12 | 2 | 26 | 24 |
| 9 | 7 | 100 | 10 | 0.5 | 23 | 21 |
| 10 | 5 | 100 | 12 | 0.5 | 21 | 18 |
| 11 | 7 | 100 | 14 | 0.5 | 25 | 23 |
| 12 | 5 | 100 | 12 | 2 | 22 | 19 |
| 13 | 7 | 100 | 12 | 1.25 | 26 | 26 |
| 14 | 9 | 100 | 10 | 1.25 | 23 | 22 |
| 15 | 5 | 50 | 12 | 1.25 | 19 | 17 |
| 16 | 7 | 100 | 12 | 1.25 | 26 | 25 |
| 17 | 7 | 150 | 10 | 1.25 | 26 | 26 |
| 18 | 7 | 100 | 12 | 1.25 | 26 | 25 |
| 19 | 5 | 100 | 10 | 1.25 | 20 | 19 |
| 20 | 9 | 100 | 12 | 0.5 | 21 | 19 |
| 21 | 7 | 150 | 12 | 0.5 | 26 | 25 |
| 22 | 7 | 150 | 14 | 1.25 | 27 | 26 |
| 23 | 7 | 150 | 12 | 2 | 28 | 27 |
| 24 | 9 | 50 | 12 | 1.25 | 22 | 20 |
| 25 | 5 | 150 | 12 | 1.25 | 21 | 19 |
| 26 | 7 | 50 | 10 | 1.25 | 21 | 19 |
| 27 | 7 | 50 | 12 | 2 | 20 | 18 |
| 28 | 7 | 100 | 12 | 1.25 | 23 | 21 |
| 29 | 7 | 100 | 10 | 2 | 25 | 24 |

Based on Plackett-Burman design incubation time, pH, starch and agitation were selected for further optimization using response surface methodology. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of –2 to +2 in relation to production of antimicrobial compounds (Table-3). As shown in Table 3, The F-value of the model was 6.05 for antibiotic activity yield, it implied that the model was very significant, and there was only a 0.01% chance that a "Model F-Value" could occur due to noise. Mo3 reover, the *P*-values (<0.0001) of the model and the lack of fit (0.7893) also suggested that the obtained experimental data was a good fit with the model. The value of determination coefficient R2= 0.8581 for antibiotic production, ensured a satisfactory adjustment of the quadratic model to the experimental data, and also indicated a high correlation between the predict values and the practical values. Normally, a regression model having an R2 value higher than 0.9 is considered and a model with an R2 value between 0.7 and 0.9 is considered as having a high correlation[15]. Significance of model was further supported by statistically insignificant lack of fit, as was evident from the lower calculated -value (1.16). Accuracy of the model can be checked by the determination of coefficient. The closer the value of to 1, the stronger the model to predict the response[18]. The quality of fit of the model was checked by coefficient of determination (R2).

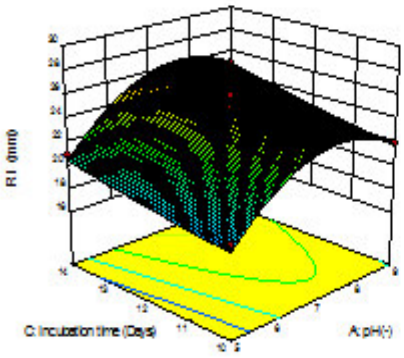
**Table 3**
***Analysis of model for antibiotic production by ANNOVA***

| Source | Sum of Squares | Df | Mean Square | F Value | P Value Prob > F |
|---|---|---|---|---|---|
| Model | 250.76 | 14 | 17.91 | 6.05 | 0.0009 |
| A-pH | 48.00 | 1 | 48.00 | 16.21 | 0.0012 |
| B-Agitation | 60.75 | 1 | 60.75 | 20.52 | 0.0005 |
| C-Incubation time | 21.33 | 1 | 21.33 | 7.21 | 0.0178 |
| D-Starch | 6.75 | 1 | 6.75 | 2.28 | 0.1533 |
| AB | 6.25 | 1 | 6.25 | 2.11 | 0.1683 |
| AC | 0.25 | 1 | 0.25 | 0.084 | 0.7756 |
| AD | 4.00 | 1 | 4.00 | 1.35 | 0.2645 |
| BC | 9.00 | 1 | 9.00 | 3.04 | 0.1032 |
| BD | 16.00 | 1 | 16.00 | 5.40 | 0.0356 |
| CD | 0.25 | 1 | 0.25 | 0.084 | 0.7756 |
| A^2 | 72.43 | 1 | 72.43 | 24.46 | 0.0002 |
| B^2 | 1.41 | 1 | 1.41 | 0.48 | 0.5010 |
| C^2 | 0.16 | 1 | 0.16 | 0.055 | 0.8181 |

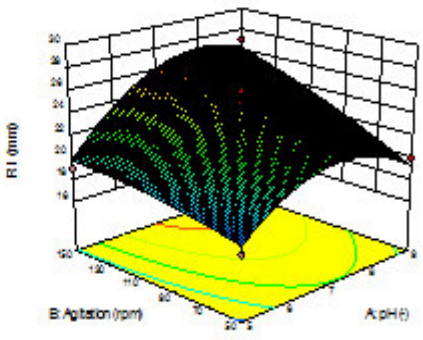
| | | | | | |
|---|---|---|---|---|---|
| D^2 | 6.06 | 1 | 6.06 | 2.05 | 0.1744 |
| Residual | 41.45 | 14 | 2.96 | | |
| Lack of Fit | 24.25 | 10 | 2.43 | 0.56 | 0.7893 |
| Pure Error | 17.20 | 4 | 4.30 | 6.05 | |
| Cor Total | 292.21 | 28 | | 16.21 | |

The three-dimensional (3D) response surface plots were drawn to illustrate the individual and interactive effects of agitation, pH, starch and incubation time on antibiotic production by *Nocardiopsis prasina* Fig 2. Each 3D plot presented the effects of two variables while the rest one was held at middle level. There was insignificant mutual interaction between agitation, pH, starch and incubation time (Figure 2).

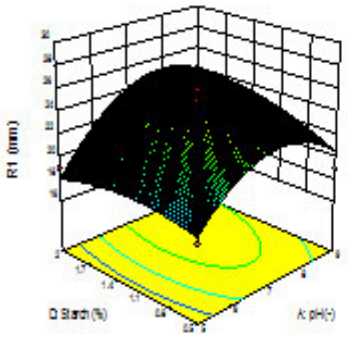
**Figure 2**
**Response surface 3D plots showing individual and interactive effects of variables on antibacterial activity of *Nocardiopsis prasina* ACT 24**



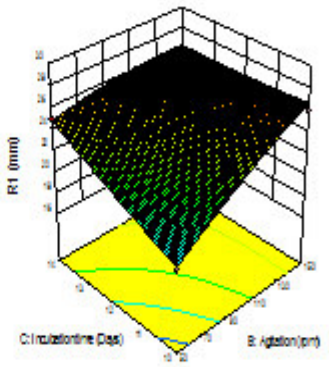

Effects of incubation time and pH



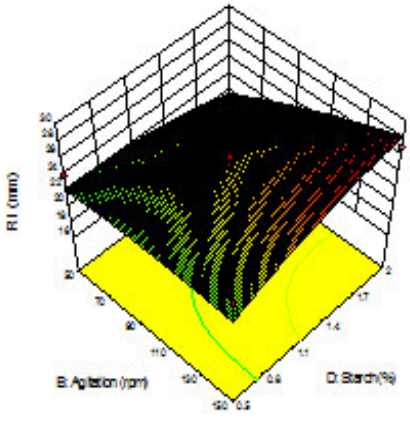

Effects of agitation and pH



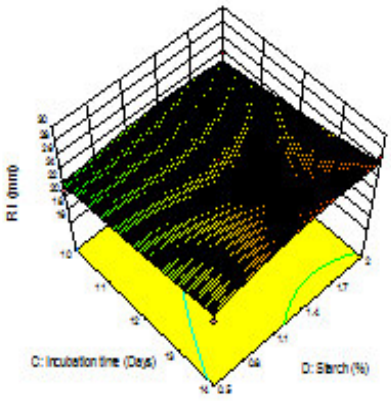

Effects of starch and pH




Effects of incubation time and agitation




Effects of agitation and starch




Effects of incubation time and starch
Validation model

The maximum experimental response for antibiotic production was 27mm whereas the predicted value was 28mm indicating a strong agreement between them. The result of optimization study under flask conditions was 29mm was observed in the scale up study with higher volume of fermentation. All the critical variables having greatest effect on the production of antibacterial compound from marine *Streptomyces* species PUA2. Optimization of process parameters resulted in increase in antibacterial activity from 7 mm to 14 mm[19].

## CONCLUSION

Present investigation focused primarily on improved production of antibiotic by *Nocardiopsis prasina* ACT 24 as a function of various levels of ingredients in production medium. PBD and RSM were found to be very effective in selecting and optimizing the medium components in manageable number of experimental trials increase the antibiotic activity. Moreover, the optimum culture medium obtained in this central composite design will be useful for further study with large scale fermentation in a fermenter to reduce cost and enhance the efficient production of antibiotic from *Nocardiopsis prasina* ACT 24.

## CONFLICT OF INTEREST

Conflict of interest declared none.

## REFERENCES


1. Arul Jose P, Jebakumar SR. Phylogenetic appraisal of antagonistic, slow growing actinomycetes isolated from hypersaline inland solar salterns at Sambhar salt Lake, IFmicb. 2013 Jul 10;4:190.
2. Shekar P, Kumar KS, Jabasingh SA, Radhakrishnan M, Balagurunathan R. Optimization of medium components for antibacterial metabolite production from marine Streptomycetes sp. Pua2 using response surface methodology. Int J Pharm Pharma Sci. 2014;6:475-80.
3. Singh D, Kaur G. Optimization of different process variables for the production of an indolizidine alkaloid, swainsonine from Metarhizium anisopliae. J.Basic Microbiol. 2012 Oct 1;52(5):590-7.
4. Montgomery DC, Myers RH. Response surface methodology: process and product optimization using designed experiments. Raymond H. Meyers and Douglas C. Montgomery. A Wiley-Interscience Publications. 1995 Jan.
5. Ghodke SK, Ananthanarayan L, Rodrigues L. Use of response surface methodology to investigate the effects of milling conditions on damaged starch, dough stickiness and chapatti quality. Food Chem. 2009 Feb 15;112(4):1010-5.
6. Song Q, Huang Y, Yang H. Optimization of fermentation conditions for antibiotic production by Actinomycetes YJ1 strain against Sclerotinia sclerotiorum. J.Agri. Sci. 2012 May 21;4(7):95.
7. Maxwell PW, Chen G, Webster JM, Dunphy GB. Stability and activities of antibiotics produced during infection of the insect Galleria mellonella by two isolates of Xenorhabdus nematophilus.AEM. 1994 Feb 1;60(2):715-21.
8. Wang Y, Fang X, An F, Wang G, Zhang X. Improvement of antibiotic activity of Xenorhabdus bovienii by medium optimization using response surface methodology. Microbial cell factories. 2011 Nov 14;10(1):98.
9. Baoxin Z, Xiangjing W, Wensheng X. Optimization of fermentation medium for enhanced production of milbemycin by a mutant of Streptomyces bingchenggensis BC-X-1 using response surface methodology. Afri.J.Biotech. 2011;10(37):7225-35.
10. Robert C, Devillers T, Wathelet B, Van Herck JC, Paquot M. Use of a Plackett− Burman Experimental Design to Examine the Impact of Extraction Parameters on Yields and Compositions of Pectins Extracted from Chicory Roots (Chicorium intybus L.).JAFC. 2006 Sep 20;54(19):7167-74.
11. Chen H, Wu MB, Chen ZJ, Wang ML, Lin JP, Yang LR. Enhancing production of a 24-membered ring macrolide compound by a marine bacterium using response surface methodology. J. Zhejiang Univ. Sci. 2013 Apr 1;14(4):346-54.
12. Usha R, Mala KK, Venil CK, Palaniswamy M. Screening of actinomycetes from mangrove ecosystem for L-asparaginase activity and optimization by response surface methodology. Polish J Microbiol. 2011 Jan 1;60(3):213-21.
13. Sharma DC, Satyanarayana T. A marked enhancement in the production of a highly alkaline and thermostable pectinase by Bacillus pumilus dcsr1 in submerged fermentation by using statistical methods. Bioresour Technol.. 2006 Mar 31;97(5):727-33.
14. Li Y, Liu Z, Cui F, Liu Z, Zhao H. Application of Plackett–Burman experimental design and Doehlert design to evaluate nutritional requirements for xylanase production by Alternaria mali ND-16. Appl Microbiol Biotechnol . 2007 Nov 1;77(2):285-91.
15. Sweetline C, Usha R, Palaniswamy M. Studies on anticandidal activity of the actinomycetes isolated from Coimbatore region of Tamil Nadu. Int J Pharm Bio Sci. 2014;5(3):46-53.
16. Rajeshkumar S, Jobitha GG, Malarkodi C, Kannan C, Annadurai G. Optimization of Marine Bacteria Enterococcus sp. Biomass Growth by using Response Surface

Methodology. J. Environ. Nanotechnol. 2013;2(1):20-7.
17. Shekar P, Kumar KS, Jabasingh SA, Radhakrishnan M, Balagurunathan R. Optimization of medium components for antibacterial metabolite production from marine Streptomycetes sp. Pua2 using response surface methodology. Int J Pharm Pharma Sci. 2014;6:475-80.
18. Bandi S, Kim Y, Chang YK, Shang G, Yu TW, Floss HG. Construction of asm2 deletion mutant of Actinosynnema pretiosum and medium optimization for ansamitocin P-3 production using statistical approach. J Microbiol Biotechnol. 2006;16(9):1338-46.
19. Ramasamy S, Balakrishna HS, Selvaraj U, Uppuluri KB. Production and Statistical Optimization of Oxytetracycline from Streptomyces rimosus NCIM 2213 using a New Cellulosic Substrate, Prosopis juliflora. BioResources. 2014 Oct 15;9(4):7209-21.

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